DRAFT OECD GUIDELINE FOR THE TESTING OF CHEMICALS

In vivo Mammalian Alkaline Comet Assay

INTRODUCTION

1. The alkaline Comet (single cell gel electrophoresis) assay is used for the detection of primary DNA damage induced in isolated cells or nuclei from multiple tissues of animals, usually rodents.

2. The purpose of the Comet assay is to identify substances that cause DNA damage. Under alkaline conditions, the Comet assay can detect single and double stranded breaks, resulting, for example, from direct interactions with DNA, alkali labile sites or as a consequence of incomplete excision repair. Under certain modified conditions the assay can detect DNA-DNA and DNA-protein crosslinking, and oxidized bases. The Comet assay has been reviewed and recommendations have been published by various expert groups (1) (2) (3) (4) (5) (6) (7) (8) (9) (10).

3. A formal validation of the *in vivo* rodent Comet assay was recently (2006-2012) coordinated by the Japanese Center for the Validation of Alternative Methods (JaCVAM), in conjunction with the European Centre for the Validation of Alternative Methods (ECVAM) and the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM)(11). This Test Guideline includes the recommended use and limitations of the Comet assay, and is based on the Comet assay method protocol version 14.2, which was ultimately developed during this validation study, and on additional relevant published and in-house data.

4. Definitions of key terms are set out in Annex 1.

INITIAL CONSIDERATIONS

5. The Comet assay is a method for measuring primary DNA strand breaks in eukaryotic cells. Single cells embedded in agarose on a microscope slide, or other suitable gel support platform, are lysed with detergent and high salt to form nucleoids containing supercoiled loops of DNA linked to the nuclear matrix. Electrophoresis at high pH results in structures resembling comets, observed by fluorescence microscopy; the intensity of the comet tail relative to the head reflects the amount of DNA breakage. (12) (13) (14).

6. The *in vivo* alkaline Comet assay is especially relevant to assessing genotoxic hazard in that the assay's responses are dependent upon *in vivo* metabolism, kinetics, and DNA repair processes, although these may vary among species, among tissues and among the types of DNA damage. The Comet assay is most often performed in rodents, although it can be applied to other species. The use of other species should be scientifically and ethically justified on a case-by-case basis. To fulfill animal welfare requirements, this assay can also be integrated with other toxicological studies, e.g., repeated dose toxicity studies (10) (31) (32), or the endpoint can be combined with other genotoxicity endpoints such as the in vivo mammalian micronucleus assay (33) (34) (35).

7. The assay has been most extensively validated in somatic tissues of male rats in collaborative studies such as the JaCVAM trial and in Rothfuss et al. 2010 (10). Nevertheless, the technique can be applied in any (mammalian) species, if justified. The liver and stomach were used in the JaCVAM international validation study, because the liver is the most active organ in metabolism of chemicals and also frequently a target organ for carcinogenicity, and the stomach is usually first site of contact for chemicals after oral exposure. However, the technique is in principle applicable to any tissue from which analyzable single cell suspensions can be derived. There are many publications using organs or tissues other than liver and stomach, e.g., jejunum (15), kidney (16) (17), trachea (18), skin (19) (20)**,** or urinary bladder (21) (22), and tests have also been performed in multiple organs (23) (24). However, the alkaline Comet assay as described in this guideline is considered not to be appropriate for studies of all stages of germ cells (see paragraph 27). The selection of route of exposure and tissue(s) to be studied should be determined based on all available/existing knowledge of the test substance e.g.

metabolism and distribution, potential for site-of-contact effects, structural alerts, other genotoxicity or toxicity data etc. Tissue(s) from treated animals can be investigated based on existing knowledge of the test substance or purpose of the study. Thus, where appropriate, the genotoxic potential of the test substance can be assayed in the target tissue(s) of carcinogenic and/or other toxic effects. If there is evidence that the test substance, or a relevant metabolite, will not reach any of the tissues of interest, it is not appropriate to perform an *in vivo* Comet assay in that tissue.

8. Further modifications of the standard alkaline assay as described in this guideline are required/recommended for efficient and specific detection of DNA cross-links or oxidised bases. For example, in order to detect DNA cross-links it may be necessary to increase the level of background DNA damage, e.g. by inducing DNA damage with a known genotoxic agent, X-ray irradiation or by increasing electrophoresis time or temperature ((25) (26) (27) in order to be able to efficiently detect reduction in DNA migration due to cross-linking activity. The addition of lesion-specific endonucleases increases the sensitivity of the Comet assay to identify certain types of oxidised bases. (28) (29) (30). The assay as described here is not designed to specifically detect aneugens.

PRINCIPLE OF THE METHOD

9. Animals are exposed to the test substance by an appropriate route once, twice or three times separated by 24 h intervals, or they can be treated for even longer period, e.g., for 28 days (10) (31) (32) . Animals are humanely killed and tissues are sampled at appropriate times after the last or only administration (see (6) and paragraph 25). The use of an early sampling time is essential because certain lesions in the DNA can be repaired quickly and efficiently (see paragraph 25). At the selected sampling time(s), the tissues of interest are dissected and single cell/nucleus suspensions are prepared (in situ perfusion may be performed where appropriate e.g. liver) and embedded in soft agar to so as to immobilize them on glass slides or other suitable gel spot support platforms. Cells/nuclei are treated with detergent to remover cellular and/or nuclear membrane, and exposed to strong alkali e.g., pH13 to allow DNA unwinding and release of DNA fragments. The nuclei in the agar are

then subjected to electrophoresis. Normal non-fragmented DNA molecules remain in the position where the nucleus had been in the agar, while fragmented DNA migrates towards the anode. After electrophoresis, the DNA is visualized using a fluorescent nucleic acid-specific stain. Preparations are analyzed using a microscope and image analysis. The extent of DNA that has migrated during electrophoresis reflects the amount of DNA breaks and the size of the fragments. There are several endpoints for the Comet assay but the DNA content in the tail (% tail DNA) is recommended to assess DNA damage (11). After analysis of sufficient nuclei, the data are analyzed with appropriate methods to judge the assay results.

VERIFICATION OF LABORATORY PROFICIENCY

10. Each laboratory should establish experimental competency in these assays by demonstrating the ability to obtain single cell or nuclei suspensions of the target tissue(s) and that the % tail DNA for vehicle treated animals are within a reproducible low range. In addition, each laboratory should be able to reproduce expected results, such as with selected chemicals from the JaCVAM validation study (11) or from other published data (see paragraph 7), for % tail DNA of positive control substances (including the ability to discriminate weak from strong responses). Initial efforts should focus on establishing proficiency with the most commonly used tissues i.e. the rodent liver where comparison with existing data and expected results may be made (11 and other published data). Data from other tissues e.g. stomach, blood etc. could be collected at the same time. The laboratory needs to demonstrate proficiency with each individual tissue they are planning to study, and will need to demonstrate that expected or predicted genotoxic effects can be obtained in that tissue. Chemicals should be selected from those listed in Table 1, although other chemicals may be selected if appropriate. Vehicle/negative control data should be collected so as to demonstrate reproducibility of negative data responses. Dose-effect relationships should also be demonstrated, where appropriate. During the course of these investigations, the laboratory can establish a historical positive control range and distribution, and a historical negative control range and distribution. Re-evaluation of laboratory proficiency is recommended if major changes to the experimental conditions are proposed for the assay.

11. The assay can be applied to any somatic tissue from which good quality suspensions

of single cells/nuclei can be prepared; some stages of germ cells are considered not to be within the scope of this guideline (see paragraph 27). Different tissues may give different negative control % tail DNA values. It is therefore important to establish negative control ranges for each tissue, which should be demonstrated to be reproducible, and should be low enough to allow the discrimination of weak from strong genotoxic effects. Selection of appropriate positive control chemicals, dose ranges and experimental conditions (e.g. electrophoresis conditions) may need also to be optimised for the detection of weak positive control dose responses.

DESCRIPTION OF THE METHOD

Preparations

Selection of animal species

12. Common laboratory strains of healthy young adult rodents, are used. The choice of rodent species should be based on (i) species used in other toxicity studies (to be able to correlate data), (ii) species that developed tumors in a carcinogenicity study (when investigating the mechanism of carcinogenesis), or (iii) species with the most relevant metabolism for humans, if known. Rats are most often the experimental animals of choice as they are most widely used in toxicity assessment of chemicals. However, other species can theoretically and practically be used if ethically and scientifically justified.

Animal housing and feeding conditions

13. For rodents, the temperature in the experimental animal room ideally should be 22^{o}C (±3^{o}C). Although the relative humidity ideally should be 50-60%, it should be at least 30% and preferably not exceed 70% other than during room cleaning. Lighting should be artificial, the sequence being 12 hours light, 12 hours dark. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water. The choice of diet may be influenced by the need to ensure a suitable admixture of a test substance when administered by this route. Rodents should be housed in small groups (usually no more than five) of the same sex if no aggressive behavior is expected. Animals may be housed individually only if

scientifically justified.

Preparation of the animals

14. Healthy young adult animals (for rodents, 6-10 weeks old at start of treatment) are normally used, and are randomly assigned to the control and treatment groups. The animals are identified uniquely and acclimated to the laboratory conditions for at least five days before the start of treatment. Cages should be arranged in such a way that possible effects due to cage placement are minimized. At the commencement of the study, the weight variation of animals should be minimal and not exceed ± 20% of the mean weight of each sex.

Preparation of doses

15. Solid test substances should be dissolved or suspended in appropriate solvents or vehicles or admixed in diet or drinking water prior to dosing of the animals. Liquid test substances may be dosed directly or diluted prior to dosing. For inhalation exposures, test materials can be administered as gas, vapour, or a solid/liquid aerosol, depending on their physicochemical properties. Fresh preparations of the test substance should be employed unless stability data demonstrate the acceptability of storage and define the appropriate storage conditions.

Test Conditions

Solvent/vehicle

16. The solvent/vehicle should not produce toxic effects at the dose volumes used, and should not be suspected of chemical reaction with the test substance. If other than well-known solvents/vehicles are used, their inclusion should be supported with reference data indicating their compatibility. It is recommended that wherever possible, the use of an aqueous solvent/vehicle should be considered first.

Controls

Positive controls

17. A group of animals treated with a positive control chemical should normally be included with each test. It is not necessary to administer concurrent positive control chemicals by the same route as the test substance. However, the positive control chemicals should be known to induce DNA lesions in one or more tissues of interest for the test substance. The doses of the positive control chemicals should be selected so as to produce moderate effects that critically assess the performance and sensitivity of the assay and are consistent with concurrent positive control ranges. Examples of positive control chemicals and some of their target tissues are included in Table 1

Table 1: Examples of positive control substances and some of their target tissues

Chemicals and CAS No.
Ethyl methanesulfonate (CAS RN 62-50-0) for liver, stomach, jejunum, kidney, bladder and peripheral blood
Ethyl nitrosourea (CAS RN 759-73-9) for liver and stomach
Methyl methanesulfonate (CAS RN 66-27-3) for liver and stomach
N-Nitrosodimethylamine (CAS RN 62-75-9) for liver
N-Methyl-N'-nitro-N-nitrosoguanidine (CAS RN: 70-25-7) for stomach
Dimethylhydrazine (which form, which CAS no) for liver and colon
Streptozotocin (CAS RN 18883-66-4) for kidney
AZT
Topoisomerase II inhibitor
Oxidative mutagen (4-NQO?)
e.g. methotrexate

Negative controls

18. A group of negative control animals, treated with solvent or vehicle alone, and otherwise treated in the same way as the treatment groups, should be included with each test for every sampling time and tissue. The % tail DNA in negative control animals should be within the pre-established laboratory background range for each

individual tissue and sampling time. In the absence of historical or published control data showing that no deleterious or mutagenic effects are induced by the chosen solvent/vehicle, untreated control animals should also be included for every sampling time and tissue in order to establish acceptability of the vehicle control.

PROCEDURE

Number and Sex of Animals

19. The number of animals per group should be predetermined to be sufficient to provide the statistical power necessary to detect [at least a doubling in genotoxic effect or a significant increase with an appropriate statistical test???]. Group sizes will normally consist of a minimum of 5 animals per sex; however, if the statistical power is insufficient, the number of animals should be increased as required. Male animals should normally be used. There may be cases where testing females alone would be justified; for example, when testing human female-specific drugs, or when investigating female-specific metabolism. If there are significant differences between the sexes in terms of toxicity or metabolism, then both males and females will be required.

Treatment schedule

20. Based on observations made in the JaCVAM validation study, treatments on three consecutive days are generally considered appropriate for producing sufficient sensitivity to detect genotoxic effects, and have the advantage of allowing the assay to be combined with the micronucleus test. Treatments on only one or two consecutive days are also acceptable for detection of comets (with appropriate sampling times – see paragraph 25), but cannot easily be combined with the micronucleus test. Alternative treatment regimens may be appropriate for some evaluations, and these alternative dosing schedules should be scientifically justified in the protocol. Samples from extended dose regimens (e.g., 28-day daily dosing) are acceptable as long as the test substance gives a positive response or, for a negative study, as long as toxicity to the target tissue(s) has been demonstrated or the limit dose has been used, and dosing continued until the time of sampling. Test substances also may be administered as a split dose, i.e., two treatments on the same day separated by no more than a few hours, to facilitate administering a large

volume of material, although adjustments may need to be made to accommodate the early sampling time following the last dose (see paragraph 25)

Dose Levels

21. If a preliminary range-finding study is performed because there are no suitable data available from other relevant studies to aid in dose selection, it should be performed in the same laboratory, using the same species, strain, sex, and treatment regimen to be used in the main study. The range-finding study should be started with the most likely dose to cause toxicity, using a small number of animals (e.g. 2). If the maximum tolerated dose (MTD) cannot be defined, a further group of animals should be exposed to a higher or lower dose depending on the clinical effects of the first dose. This strategy should be repeated until the appropriate MTD is found. The highest dose level should be chosen with the aim of inducing slight toxic effects but not death or evidence of pain, suffering or distress necessitating humane euthanasia. However, doses inducing severe histopathological changes should be avoided. It may be necessary to extend the observation period beyond 2-3 days in the range-finder to see whether such severe effects lead to lack of tolerance, and therefore to avoid dosing above the MTD in the main study. For a non-toxic substance, with an administration period of 14 days or more, the maximum (limit) dose is 1000 mg/kg bodyweight/day. For administration periods of less than 14 days the maximum (limit) dose is 2000 mg/kg bodyweight/day. For certain types of test substance (e.g. human pharmaceuticals) covered by specific regulations these limits may vary.

22. Substances with specific biological activities at low non-toxic doses (such as hormones and mitogens), and substances that exhibit saturation of toxicokinetic properties may be exceptions to the dose-setting criteria and should be evaluated on a case-by-case basis.

23. In addition to the maximum dose (MTD or limit dose) a descending sequence of at least two additional appropriately spaced (less than SQR(10)) dose levels should be selected for each sampling time to demonstrate dose-related responses. Studies intending to more fully characterize the quantitative dose-response information may require additional dose groups. The dose levels used should preferably cover a range from the maximum (MTD or limit dose) to one producing little or no toxicity.

Administration of Doses

24. The anticipated route of human exposure should be considered when designing an assay. Therefore, routes of exposure such as oral (by gavage), drinking water, subcutaneous, intravenous, topical, inhalation, intratracheal, dietary, or implantation may be chosen as justified. In any case the route should be chosen to ensure adequate exposure of the target tissue(s). Intraperitoneal injection is not recommended since it is not a physiologically relevant route of human exposure. The maximum volume of liquid that can be administered by gavage or injection at one time depends on the size of the test animal. The volume should not exceed 2 mL/100g body weight. The use of volumes greater than this (if permitted by animal welfare legislation) should be justified. Variability in test volume should be minimized by adjusting the concentration of the dosing formulation to ensure a constant volume at all dose levels.

Sampling Time

25. The sampling time is a critical variable because it is determined by the period needed for DNA damage to be induced but before that damage is removed, repaired or leads to cell death. The life of some of the DNA lesions detected by the Comet assay (at least when conducted in vitro), such as alkali labile sites and single strand breaks, is very short, in the order of minutes, not hours (36) (37). Accordingly, measures should be taken to mitigate the loss of such transient DNA lesions, and the resultant reduction in assay sensitivity, by ensuring that tissues are sampled sufficiently early. The optimum sampling time(s) may be chemical- or route-specific (for example intravenous administration will likely lead to rapid tissue exposure), and sampling times should be determined from kinetic data where available. In the absence of kinetic data (for example, defining the peak plasma or tissue concentration) a suitable compromise for the measurement of genotoxicity is to sample at 2-6 h after the last treatment for two or more treatments, or at both 2-6 and 16-26 h after a single administration.

Observations

26. General clinical observations related to the health of the animals should be made

and recorded at least once a day preferably at the same time(s) each day and considering the peak period of anticipated effects after dosing (38). At least twice daily, all animals should be observed for morbidity and mortality. For longer duration studies, all animals should be weighed at least once a week, and at completion of the test period. For (sub)chronic or dietary studies, food consumption should be measured at each change of food and at least weekly. If the test substance is administered via the drinking water, water consumption should be measured at each change of water and at least weekly. Animals exhibiting non-lethal indicators of excess toxicity should be humanely euthanized prior to completion of the test period.

Tissue Collection

27. Since it is possible to study induction of DNA damage (comets) in virtually any tissue, the rationale for selection of tissues to be collected should be clearly defined and based upon the reason for conducting the study and any existing kinetic, genotoxicity, carcinogenicity or toxicity data for the test chemical under investigation. Important factors for consideration should include the route of administration (based on likely human exposure route(s)), the predicted tissue distribution, the role of metabolism and the possible mechanism of action. In the absence of any background information, those somatic tissues that may be of interest should be collected. However, it is important that the laboratory has demonstrated proficiency with those tissues. In the absence of data suggesting particular tissues of interest, sampling the liver and glandular stomach or duodenum/jejunum should be considered as these represent sites of metabolism and of direct contact (at least for orally-administered substances). It should be noted, that whilst there may be an interest in genotoxic effects in germ cells, the standard alkaline Comet assay as described in this guideline is not considered appropriate to measure DNA damage in all stages of germ cells. There are publications which have described genotoxic effects as measured by the Comet assay in some germ cell stages (50) (51) and advice on the design of such studies can be obtained from the given references.

28. Although the JaCVAM validation trial only studied isolated cells, it has been shown that there was no essential difference in the assay result whether isolated cells or nuclei were used (8). Also the method to isolate cells/nuclei (e.g., homogenizing,

mincing, enzymic digestion and mesh filtration) did not give significantly different results (39). Consequently either isolated cells or isolated nuclei can be used.

Preparation of specimens

29. For the processes described in the following paragraphs (30-37) it is important that all solutions are freshly prepared. This especially important for the lysing solution.

30. Animals will be humanely killed, consistent with animal welfare and 3Rs, at the appropriate time(s) after the last treatment with a test substance. Tissues will be dissected, a portion of the tissue will be collected for the comet assay and a section of the tissue will be cut and placed in formalin for possible histopathology analysis. The tissue for the Comet assay will be placed into ice-cold mincing buffer, rinsed sufficiently with cold mincing buffer to remove residual blood, and stored in ice-cold mincing buffer until processed. In situ perfusion may also be performed, e.g. for liver, kidney. Histopathology is usually not performed when negative findings are obtained. In the case of positive findings, fixed samples will be processed for histopathological examination according to standard methods (11).

31. Single cell/nuclei preparations should normally be made within one hour after the animals have been humanely killed. If the liver and the stomach are selected, for example, they could be processed according to the methods described in the JaCVAM trial report (11). These involved removing tissue, washing, mincing (liver) or scraping (stomach), allowing clumps to settle and straining. However other methods such as homogenization and enzymic digestion are also used to prepare single cell/nuclei suspensions from these and other tissues. As discussed in paragraph 25, the life of some of the DNA lesions detected by the Comet assay may be very short (36) (37). Therefore, whatever method is used to prepare the single cell/nuclei suspensions it is important that tissues are processed as soon as possible after sacrifice and placed in conditions that reduce the disappearance of lesions (e.g. by maintaining the tissue at low temperature or by inclusion of DNA repair inhibitors). The cell suspensions should be kept ice-cold until ready for use, and that minimal inter-sample variation and appropriate positive and negative control responses can be demonstrated.

32. Gel preparation (on slides or other platforms) should be done as soon as possible (e.g. within one hour) after single cell/nuclei preparation. Comet gels should be prepared using laboratory specific procedures. The volume of the cell suspension added to low melting point agarose (usually 0.5-1.0%) to make the gels should not reduce the percentage of low melting point agarose to less than 0.45%.

33. Once prepared, the gels should be immersed in chilled lysing solution for at least one hour (or overnight) at around 2-8^{o}C under light proof conditions. After this incubation period, the gels should be rinsed (e.g. in purified water, neutralization solution or electrophoresis buffer) to remove residual detergent and salts prior to the alkali unwinding step.

34. It should be noted that altering various aspects of the methodology, including sample preparation, electrophoresis conditions, and visual analysis parameters, have been investigated and can affect DNA migration of (40) (41) (42).

Unwinding and electrophoresis

35. Gels should be randomly placed onto the platform of a submarine-type electrophoresis unit containing sufficient electrophoresis solution such that the surfaces of the gels are completely covered. A balanced design should be used, i.e., in each electrophoresis run, there should be the same number of gels from each animal in the study. The gels should be left for at least 20 minutes for the DNA to unwind, and then subjected to electrophoresis under controlled conditions that will maximize the dynamic range of the assay (i.e. lead to acceptable levels of % tail DNA for negative and positive controls that maximize sensitivity). Based on the JaCVAM trial this could be 0.7 V/cm for at least 20 minutes, with a constant voltage delivering a starting current of 300 mA, but these conditions may need to be varied according to tissue sample, depth of electrophoresis buffer above the gels, electrophoresis equipment used, battery pack etc. The optimum conditions should therefore be determined during the demonstration of proficiency with each tissue studied. The current at the start and end of the electrophoresis period should be recorded. The temperature of the electrophoresis solution through unwinding and electrophoresis should be maintained at a temperature, usually <10^{o}C. The temperature of the electrophoresis solution at the start of unwinding, the start of electrophoresis, and the end of electrophoresis should be recorded.

36. After completion of electrophoresis, the gels should be immersed/rinsed in the neutralization buffer for at least 5 minutes. Gels can be stained and scored "fresh" (within 24 hours) or can be dehydrated for later use. However, historical data should be obtained and retained separately for each of these conditions. In case of the latter, gels should be dehydrated by immersion into absolute ethanol for at least 5 minutes, allowed to air dry, and then stored, either at room temperature or refrigerated, until scored. Once scored, gels should be retained and stored under low humidity conditions (e.g., in a desiccator) for potential rescoring.

37. Further modifications of the standard alkaline assay as described in this guideline are required/recommended for efficient and specific detection of DNA cross-links or oxidised bases. For example, in order to detect DNA cross-links it may be necessary to increase the level of background DNA damage, e.g. by inducing DNA damage with a known genotoxic agent, X-ray irradiation or by increasing electrophoresis time or temperature (25) (26) (27) in order to be able to efficiently detect reduction in DNA migration due to cross-linking activity. The addition of lesion-specific endonucleases increases the sensitivity of the Comet assay to identify certain types of oxidised bases. (28) (29) (30).

Methods of Measurement

38. All gels for analysis, including those of positive and negative controls, should be independently coded before any type of analysis and should be randomized so the scorer is unaware of the treatment condition. The gels will be stained with nucleic acid specific fluorochrome e.g., SYBR Gold, Green I, propidium iodide or ethidium bromide on the day of scoring. The comets should be measured via a digital (e.g. CCD) camera linked to an image analyzer system using a fluorescence microscope at suitable magnification, e.g., 200X. For each sample (per tissue per animal), where possible, at least 150 cells should be analyzed. If slides are used, this could be from 2 or 3 slides scored per sample when five animals per group are used. Several areas of the gel should be observed at 5 cells or less/field, taking care to avoid any selection bias or overlap counting of cells. Scoring at the edge of gels should be avoided.

39. DNA damage in the comet assay can be measured by three independent

endpoints; % tail DNA, tail length and tail moment. All three measurements can be made if the appropriate image software analyzer system is used. The % tail DNA is recommended for the evaluation and interpretation of results, and is determined by the DNA fragment intensity in the tail expressed as a percentage of the cell's total intensity (12).

40. Only cells of good morphology (clearly defined head and tail with no interference with neighbouring cells) should be scored. Artifacts should be avoided. Cells may be classified into three categories as described in the atlas of comet images (43), namely scorable, non-scorable and "hedgehog" (see paragraph 41 for further discussion). The frequency of hedgehogs should be determined based on the visual scoring of at least 100 cells per sample (see paragraph 41 for further discussion) and separately documented, but should be excluded from data analysis. The following should also be excluded from data analysis; (a) scorable cells with 90% or more DNA in the tail (42), (b) cells that appear analyzable but the recognition by software is considered incorrect (e.g. the automatic recognition of nucleus center is shifted), (c) cells where the staining of the nucleus and/or area of migration is considered poor, and (d) cells overlapping other cells in a dense area of the gel.

Tissue damage and cytotoxicity

41. Hedgehogs (or clouds) are heavily damaged cells that exhibit a microscopic image consisting of a small or non-existent head, and large diffuse tails. In a good quality experiment, the percentage of hedgehogs may reflect DNA breaks resulting from cytotoxicity, and therefore could provide useful information. However, an increase in the frequency of hedgehogs may indicate poor tissue quality rather than tissue damage. Therefore, the percentage of hedgehogs observed on slides is a useful parameter to evaluate the quality of assay. If negative control preparations show unusually high levels of hedgehogs, the impact on the reliability of the study needs to be considered. Since fragmentation of the DNA can be induced not only by chemical genotoxicity but also during the process of cell death, i.e., apoptosis and necrosis, determination of the frequency of hedgehogs alone will not allow genotoxic and cytotoxic events to be discriminated. Sometimes it is difficult to distinguish between genotoxicity and apoptosis/necrosis by the shape of the nucleus and the comet tail after electrophoresis, and investigators have to make a subjective decision. To help with these subjective decisions, an atlas of comet images, with

many examples, has been published (43). Data from the neutral diffusion assay is also sometimes collected to provide an indication of cytotoxicity, however it should be evaluated cautiously because some reports indicate it is not always reliable.

42. Histopathological changes are considered the most relevant indicator of cytotoxicity. Low or moderate cytotoxicity (histopathological observations such as inflammation, infiltration, apoptotic or necrotic changes) is often seen with known genotoxins, and make it difficult to distinguish whether such changes are due to genotoxic or cytotoxic effects. Severe cytotoxicity (seen through histopathology) might influence the biological significance of the results. For this reason, doses inducing severe histopathological changes should be avoided. As mentioned earlier, it may be necessary to extend the observation period in the range-finder to see whether such severe effects lead to lack of tolerance. It is clear that any changes in % tail DNA would require very careful interpretation if they were seen alongside severe cytotoxic changes observed through histopathology (11) (44). There are several additional indicators of tissue damage that may be considered (caspase activation, TUNEL stain, Annexin V stain, etc.) but there are limited published experiences with the use of these for in vivo studies and some may be less reliable than others. Clinical chemistry measures (e.g. AST, ALT) can also provide useful information on tissue damage.

DATA AND REPORTING

Treatment of Results

43. Individual animal data should be presented in tabular form. The experimental unit is the animal. The report should include the individual % tail DNA for each tissue from each animal.

Evaluation and Interpretation of Results

44. Negative control values should be used to judge the acceptability of each assay, and the acceptance criteria should be set in advance based on historical negative control values established for each tissue. By way of guidance for the most widely studied tissues, recommended means of % tail DNA in negative control groups are 1-8% in the liver and 1-20% in the stomach based on data collected in the JaCVAM

validation trial. Mean of negative control values below 1% are not recommended because such low values can often lead to misinterpretation of Comet assay results (10).

45. There are several criteria for determining a positive result, such as a dose-related increase in the % tail DNA or a clear statistically significant increase in the % tail DNA in a single dose group compared to the solvent/vehicle control group. At least three treated dose groups should be analysed in order to provide sufficient data for dose-response analysis. Appropriate statistical methods should be used in evaluating the test results (45) (46) (47) (48). Statistical tests used should consider the animal as the experimental unit. Providing that all acceptability criteria are fulfilled, a test substance is considered a clear positive if at least one of the dose groups treated with test substance exhibits a statistically significant increase in % tail DNA compared to the concurrent negative control, and the increase is dose-related. The test substance is then considered able to induce DNA damage in vivo. If the assay is modified to detect cross linking agents, then the dose response may exhibit a decrease.

46. Providing that all acceptability criteria are fulfilled, a test substance is considered a clear negative if none of the dose groups exhibit a statistically significant increase in % tail DNA compared to the concurrent negative control, and if there is no dose-related increase. The test substance is then considered unable to induce DNA damage in vivo.

47. There is no requirement for verification of a clear positive or negative response. However, in case the response is not clearly negative or positive, and in order to assist in establishing the biological relevance of a result, the data should be evaluated by expert judgement and/or further investigations using the existing data, such as determining if the positive result is outside the distribution of the historical negative control data (e.g. 95% confidence interval) (49).

48. For biological relevance of a positive result, cytotoxicity at the target tissue should also be discussed. Histopathological information can help in the interpretation of a positive result in the Comet assay (see paragraph 42). Careful interpretation of increased or decreased % tail DNA in the presence of severe cytotoxicity is therefore essential.

49. Positive results in the Comet assay indicate that the test substance induces DNA damage in the specific tissues of the test species. Negative results indicate that, under the test conditions, the test substance does not induce DNA damage in the specific tissues of the test species.

Test Report

50. The test report should include the following information:

Test substance:
- identification data and CAS RN, if known;
- source, lot number if available;
- physical nature and purity;
- physicochemical properties, if relevant to the conduct of the study;
- stability of the test substance, if known;

Solvent/vehicle:
- justification for choice of solvent/vehicle;
- solubility and stability of the test substance in the solvent/vehicle, if known;
- preparation of dose formulations;
- analytical determinations on formulations (e.g., stability, homogeneity, nominal concentrations);

Test animals:
- species/strain used and justification for the choice;
- number, age and sex of animals;
- source, housing conditions, diet, etc.;
- individual weight of the animals at the start of the test, including body weight range, mean and standard deviation for each group;

Test conditions:
- positive and negative (vehicle/solvent) control data;
- data from the range-finding study (if conducted);
- rationale for dose level selection;
- details of test substance preparation;
- details of the administration of the test substance;

- rationale for route of administration;
- methods for sample preparation, where available, histopathological analyses, especially for a substance giving a positive comet response
- methods for verifying that the test substance reached the target tissue, or general circulation, if negative results are obtained;
- actual dose (mg/kg body weight/day) calculated from diet/drinking water test substance concentration (ppm) and consumption, if applicable;
- details of food and water quality;
- detailed description of treatment and sampling schedules and justifications for the choices (e.g. toxicokinetic data, where available);
- method of euthanasia;
- procedures for isolating and preserving tissues;
- methods for preparing single cell/nucleus suspension;
- source and lot numbers of all reagents (where possible);
-
- methods for evaluating cytotoxicity; - electrophoresis conditions;
- staining techniques used; and
- methods for scoring and measuring comets:

Results:
- animal visible condition prior to and throughout the test period, including visible signs of test substance toxicity;
- evidence of cytotoxicity if performed
- body and relevant organ weights at euthanasia;
- dose-response relationship, where evident;
- for each tissue/animal, the % tail DNA and mean/median values per group;
- concurrent and historical negative control data with ranges, means/medians and standard deviations for each tissue evaluated;
- concurrent positive control (or non-concurrent DNA positive control) data;
- statistical analyses and methods applied; and
- criteria for considering a response as positive, negative or equivocal:

Discussion of the results

Conclusion

LITERATURE

(1) Kirkland D, Speit G. Evaluation of the ability of a battery of three *in vitro* genotoxicity tests to discriminate rodent carcinogens and non-carcinogens III. Appropriate follow-up testing *in vivo*. Mutat. Res. 2008; 654:114-32.

(2) Brendler-Schwaab S, Hartmann A, Pfuhler S, Speit G., The in vivo comet assay: use and status in genotoxicity testing. Mutagenesis 2005; 20:245-54.

(3) Burlinson B, Tice RR, Speit G, Agurell E, Brendler-Schwaab SY, Collins AR, Escobar P, Honma M, Kumaravel TS, Nakajima M, Sasaki YF, Thybaud V, Uno Y, Vasquez M, Hartmann A. Fourth International Workgroup on Genotoxicity Testing: result of the in vivo comet assay workgroup. Mutat. Res. 2007; 627:31-5.

(4) Burlinson B. The in vitro and in vivo comet assays, Methods Mol. Biol. 2012; 817: 143-63.

(5) Smith CC, Deborah J. Adkins, Elizabeth A. Martin, and Michael R. O'Donovan, Recommendations for design of the rat comet assay, Mutagenesis 2008; 23:233-40.

(6) Hartmann A, Agurell E, Beevers C, Brendler-Schwaab S, Burlinson B, Clay P, Collins A, Smith A, Speit G, Thybaud V, Tice RR. Recommendations for conducting the in vivo alkaline Comet assay. Mutagenesis 2003; 18:45-51.

(7) McKelvey-Martin VJ, Green MH, Schmezer P, Pool-Zobel BL, De Méo MP, Collins A., The single cell gel electrophoresis assay (comet assay): a European review. Mutat. Res. 1993; 288:47-63.

(8) Tice RR, Agurell E, Anderson D, Burlinson B, Hartmann A, Kobayashi H, Miyamae Y, Rojas E, Ryu JC, Sasaki YF. Single cell gel/Comet assay: guidelines for in vitro and in vivo genetic toxicology testing. Environ. Mol. Mutagen. 2000; 35:206-21.

(9) Singh NP, McCoy MT, Tice RR, Schneider EL. A simple technique for quantitation of low levels of DNA damage in individual cells. Exp. Cell Res. 1988; 175:184-91.

(10) Rothfuss A, O'Donovan M, De Boeck M, Brault D, Czich A, Custer L, Hamada S, Plappert-Helbig U, Hayashi M, Howe J, Kraynak AR, van der Leede B-J, Nakajima M, Priestley C, Thybaud V, Saigo K, Sawant S, Shi J, Storer R, Struwe M, Vock E, Galloway S. Collaborative study on fifteen compounds in the rat-liver Comet assay integrated into 2- and 4-week repeat-dose studies. Mutat Res. 2010; 702:40-69.

(11) JaCVAM report

(12) Olive PL, Banath JP, Durand RE. Heterogeneity in radiation-induced DNA damage and repair in tumor and normal cell using the “comet” assay. Radiat. Res. 1990; 122:86-94.

(13) Tice RR, Strauss GH., The single cell gel electrophoresis/comet assay: a potential tool for detecting radiation-induced DNA damage in humans. Stem Cells 1995; 13 Suppl 1:207-14.

(14) Collins AR. The comet assay for DNA damage and repair: principles, applications, and limitations. Mol Biotechnol. 2004; 26:249-61.

(15) Hartmann A, Schumacher M, Plappert-Helbig U, Lowe P, Suter W, Mueller L. Use of the alkaline in vivo Comet assay for mechanistic genotoxicity investigations. Mutagenesis 2004; 19:51-9.

(16) Nesslany F, Zennouche N, Simar-Meintières S, Talahari I, NKili-Mboui EN, Marzin D. In vivo Comet assay on isolated kidney cells to distinguish genotoxic carcinogens from epigenetic carcinogens or cytotoxic compounds. Mutat. Res. 2007; 630:28-41.

(17) Brendler-Schwaab SY, Herbold BA. A new method for the enrichment of single renal proximal tubular cells and their first use in the comet assay. Mutat Res. 1997; 393:175-8.

(18) Naya M, Kobayashi N, Endoh S, Maru J, Honda K, Ema M, Tanaka J, Fukumuro M, Hasegawa K, Nakajima M, Hayashi M, Nakanishi J. In vivo genotoxicity study of single-wall carbon nanotubes using comet assay following intratracheal

instillation in rats. Regul. Toxicol. Pharmacol. 2012; 64:124-129.

(19) Toyoizumi T, Ohta R, Nakagawa Y, Tazura Y, Kuwagata M, Noguchi S, Yamakage K. Use of the in vivo skin comet assay to evaluate the DNA-damaging potential of chemicals applied to the skin., Mutat Res. 2011; 726:175-80.

(20) Struwe M, Greulich KO, Junker U, Jean C, Zimmer D, Suter W, Plappert-Helbig U. Detection of photogenotoxicity in skin and eye in rat with the photo comet assay. Photochem. Photobiol. Sci. 2008; 7:240-9.

(21) Wada K, Ohnuma A, Kojima S, Yoshida T, Matsumoto K. A comparison of cell-collecting methods for the Comet assay in urinary bladders of rats. Mutat. Res. 2012; 742:26-30.

(22) Wang A, Robertson JL, Holladay SD, Tennant AH, Lengi AJ, Ahmed SA, Huckle WR, Kligerman AD. Measurement of DNA damage in rat urinary bladder transitional cells: improved selective harvest of transitional cells and detailed Comet assay protocols. Mutat. Res. 2007; 634:51-9.

(23) Sasaki YF, Sekihashi K, Izumiyama F, Nishidate E, Saga A, Ishida K, Tsuda S. The comet assay with multiple mouse organs: comparison of comet assay results and carcinogenicity with 208 chemicals selected from the IARC monographs and U.S. NTP Carcinogenicity Database. Crit. Rev. Toxicol. 2000; 30:629-799.

(24) Sekihashi K, Yamamoto A, Matsumura Y, Ueno S, Watanabe-Akanuma M, Kassie F, Knasmüller S, Tsuda S, Sasaki YF. Comparative investigations of multiple organs of mice and rats in the comet assay. Mutat. Res. 2002; 517:53-74.

(25) Merk O, Speit G. Detection of crosslinks with the comet assay in relationship to genotoxicity and cytotoxicity. Environ. Mol. Mutagen. 1999; 33:167-72.

(26) Pfuhler S, Wolf HU. Detection of DNA-crosslinking agents with the alkaline comet assay. Environ. Mol. Mutagen. 1996; 27:196-201.

(27) Wu JH, Jones NJ. Assessment of DNA interstrand crosslinks using the modified alkaline comet assay. Methods Mol. Biol. 2012; 817:165-81.

(28) Møller P. The alkaline comet assay: towards validation in biomonitoring of DNA damaging exposures. Basic Clin. Pharmacol. Toxicol. 2006; 98:336-45.

(29) Fairbairn DW, Olive PL, O'Neill KL. The comet assay: a comprehensive review. Mutat. Res. 1995; 339:37-59.

(30) Klaude M, Eriksson S, Nygren J, Ahnström G. The comet assay: mechanisms and technical considerations. Mutat Res. 1996; 363:89-96.

(31) Rothfuss A, Honma M, Czich A, Aardema MJ, Burlinson B, Galloway S, Hamada S, Kirkland D, Heflich RH, Howe J, Nakajima M, O'Donovan M, Plappert-Helbig U, Priestley C, Recio L, Schuler M, Uno Y, Martus HJ. Improvement of in vivo genotoxicity assessment: combination of acute tests and integration into standard toxicity testing. Mutat Res. 2011; 723:108-20.

(32) Kushwaha S, Tripathi DN, Vikram A, Ramarao P, Jena GB. Evaluation of multi-organ DNA damage by comet assay from 28 days repeated dose oral toxicity test in mice: A practical approach for test integration in regulatory toxicity testing. Regul. Toxicol. Pharmacol. 2010; 58:145–54.

(33) Vasquez MZ. Combining the in vivo comet and micronucleus assays: a practical approach to genotoxicity testing and data interpretation. Mutagenesis 2010; 25:187-99.

(34) Bowen DE, Whitwell JH, Lillford L, Henderson D, Kidd D, McGarry S, Pearce G, Beevers C, Kirkland DJ. Evaluation of a multi-endpoint assay in rats, combining the bone-marrow micronucleus test, the Comet assay and the flow-cytometric peripheral blood micronucleus test**.** Mutat. Res. 2011; 722:7-19.

(35) Recio L, Hobbs C, Caspary W, Witt KL., Dose-response assessment of four genotoxic chemicals in a combined mouse and rat micronucleus (MN) and Comet assay protocol. J. Toxicol. Sci. 2010; 35:149-62.

(36) Blakey DH, Douglas GR. Transient DNA lesions induced by benzo[a]pyrene in Chinese hamster ovary cells. Mutat. Res. 1984; 140:141-45.

(37) Blakey DH, Douglas GR. The role of excision repair in the removal of transient benzo[a]pyrene-induced DNA lesions in Chinese hamster ovary cells. Mutat. Res. 1990; 236:35-41.

(38) OECD Guidance Document on the Recognition, Assessment and Use of Clinical Signs as Human Endpoints for Experimental Animals Used in Safety Evaluation, OECD Series on Testing and Assessment, No. 19, 2002, OECD Publishing.

(39) Nakajima M, Ueda M, Yamakage K, Nakagawa Y, Nakagawa M, Ohyama W, Omori T, Asano N, Hayashi M, Uno Y. Tissue sample preparation for in vivo rodent alkaline comet assay. Genes Environ. 2012; 34:50-4.

(40) Ersson C, Möller L. The effects on DNA migration of altering parameters in the comet assay protocol such as agarose density, electrophoresis conditions and durations of the enzyme or the alkaline treatments. Mutagenesis 2011; 26:689-95.

(41) Møller P, Möller L, Godschalk RWL, Jones GDD. Assessment and reduction of comet assay variation in relation to DNA damage: studies from the European Comet Assay Validation Group. Mutagenesis 2010; 25:109-11.

(42) Forchhammer L, Johansson C, Loft S, Möller L, Godschalk RWL, Langie SAS, Jones GDD, Kwok RWL, Collins AR, Azqueta A, Phillips DH, Sozeri O, Stępnik M, Palus J, Vogel U, Wallin H, Routledge MN, Handforth C, Allione A, Matullo G, Teixeira JP, Costa S, Riso P, Porrini M, Møller P. Variation in the measurement of DNA damage by comet assay measured by the ECVAG inter-laboratory validation trial. Mutagenesis 2010; 25:113-23.

(43) Comet Atlas

(44) Burlinson B, Tice RR, Speit G, Agurell E, Brendler-Schwaab SY, Collins AR, Escobar P, Honma M, Kumaravel TS, Nakajima M, Sasaki YF, Thybaud V, Uno Y, Vasquez M, Hartmann A. Fourth International Workgroup on Genotoxicity Testing: result of the in vivo comet assay workgroup. Mutat. Res. 2007; 627:31-5.

(45) Lovell DP, G Thomas G, R Dubow. Issues related to the experimental design

and subsequent statistical analysis of in vivo and in vitro comet studies. Teratog. Carcinog. Mutagen. 1999; 19:109-19.

(46) Wiklund SJ, E Agurell. Aspects of design and statistical analysis in the Comet assay. Mutagenesis 2003; 18:167-75.

(47) Bright J, Aylott M, Bate S, Geys H, Jarvis P, Saul J, Vonk R. Recommendations on the statistical analysis of the Comet assay. Pharm. Stat. 2011; 10:485-93.

(48) Lovell DP, Omori T. Statistical issues in the use of the comet assay. Mutagenesis 2008; 23:171-82

(49) Hayashi M, Dearfield K, Kasper P, Lovell D, Martus HJ, Thybaud V. Compilation and use of genetic toxicity historical control data. Mutat. Res. 2011; 723:87-90.

(50) Zheng H, Olive PL. Influence of oxygen on radiation-induced DNA damage in testicular cells of C3H mice. Int J Radiat Biol. 71:275-282. 1997

(51) Cordelli E, Fresegna AM, Leter G, Eleuteri P, Spano M, Villani P. Evaluation of DNA damage in different stages of mouse spermatogenesis after testicular X irradiation. Radiat Res. 160:443-451. 2003.

Annex 1 DEFINITIONS – *to be completed*

Alkaline single cell gel electrophoresis:
Comet:
Predictivity:
UDS:
% tail DNA

Annex 2 Solutions

The following are examples which were used in JaCVAM trials. Other preparation methods are acceptable if justified.

1) **Agarose**

1.0-1.5% (w/v) standard agarose gel for the bottom layer (if used) Regular melting agarose will be dissolved at 1.0-1.5% (w/v) in Dulbecco's phosphate buffer (Ca^{++}, Mg^{++} free and phenol free) by heating in a microwave.

0.5 % (w/v) low-melting agarose (Lonza, NuSieve GTG Agarose) gel for the cell-containing layer and, if used, a top layer Low-melting agarose will be dissolved at 0.5% (w/v) in Dulbecco's phosphate buffer (Ca^{++}, Mg^{++} free and phenol free) by heating in a microwave. During the study this solution will be kept at 37-45°C and discarded afterward.

2) Lysing solution

The lysing solution will consist of 100 mM EDTA (disodium), 2.5 M sodium chloride, and 10 mM tris hydroxymethyl aminomethane in purified water, with the pH adjusted to 10.0 with 1 M sodium hydroxide and/or hydrochloric acid. This solution may be refrigerated at <10°C until use. On the same day of use, 1 % (v/v) of triton-X100 and 10 % (v/v) DMSO will be added to this solution and the complete lysing solution will be refrigerated at <10°C for at least 30 minutes prior to use.

3) Alkaline solution for unwinding and electrophoresis

The alkaline solution consists of 300 mM sodium hydroxide and 1 mM EDTA (disodium) in purified water, pH >13. This solution will be refrigerated at <10°C until use. The pH of the solution will be measured just prior to use.

4) Neutralization solution

The neutralization solution consists of 0.4 M tris hydroxymethyl aminomethane in purified water, pH 7.5. This solution will be either refrigerated at <10°C or stored consistent with manufacturer's specifications until use.

5) Mincing buffer

The mincing buffer consists of 20 mM EDTA (disodium) and 10% DMSO in Hank's Balanced Salt Solution (HBSS) (Ca^{++}, Mg^{++} free, and phenol red free if available), pH 7.5 (DMSO will be added immediately before use). This solution will be refrigerated at <10°C until use.

6) **Staining solution**

The fluorescent DNA stain is SYBR Gold (Invitrogen-Molecular Probes), prepared and used according to the manufacturer's specifications.